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Detection, isolation and identification of more bioactive compounds from *Fusarium equiseti,* an endophytic fungus isolated from *Ocimum gratissimum*

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Abstract

Endophytes have continued to gain fame due to their ability to produce an array of secondary metabolites within the host system with huge untapped pharmacological potentials. This study was carried out to further identify and isolate novel therapeutic compounds from *Fusarium equiseti,* an Endophytic fungus isolated from leaves of *Ocimum gratissimum*. Endophytic fungal isolation, fungal fermentation, and extraction of secondary metabolites were carried out using standard laboratory methods. The crude extracts of *Fusarium equiseti* were subjected to further chromatographic techniques using vacuum liquid chromatography, Sephadex LH 20 and semipreparative HPLC for isolation of bioactive compounds. The fractions and the isolated compounds obtained were further subjected to high performance liquid chromatography-Diode Array Detector (HPLC-DAD). The analytical HPLC led to the further detection of many bioactive compounds namely: Enniatin A, Aureonitol, Serasinoside H1, Altenusin, Aplysinamisin, benzylnitril, ruspolinone and Orientin. Semipreparative HPLC led to the isolation of 6 pure compounds of which two were identified as benzylnitril, and ruspolinone. The remaining four were not identified due to lack of library hits. The detected and the isolated compounds have been previously shown to exhibit a wide array of biological activities including antiviral, antifungal, hepatoprotection, antibacterial, anticancer, cytotoxic, and antioxidant properties. The unidentified compounds may hold enormous potential as new bioactive lead compounds for development into novel therapeutic agents. Therefore, the Endophytic fungus, *Fusarium equiseti* should be harnessed for its potential pharmacological, pharmaceutical, agricultural and industrial applications.

Keywords: Endophytic fungi; *Fusarium equiseti*; *Ocimum gratissimum*; HPLC; Chromatogram; Compounds

1. Introduction

Ocimum gratissimum L (*O. gratissimum)* is an aromatic herb usually found in the tropics and is commonly known as Camphor basil or Ram Tulsi with a characteristic clove-like flavor belonging to the family of Lamiaceae. It has been reported to have for wide range of therapeutic applications, such as chemo-preventive, anti-carcinogenic, free radical

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scavenging, and radio defensive [1] and antimicrobial activities with numerous other uses across different cultures where they are found.

Numerous phytochemicals of therapeutic importance have been reported in previous studies by other researchers, for instance [2] using dried leaf of *O. gratissimum* reported the following phytochemicals such as saponins, alkaloids, phenols, phlobatanins, and glycosides and this could be the justification for its numerous usages for the treatment of various diseases traditionally.

Previous studies suggest that endophytes possess the ability to produce the same or similar chemicals as those originating from their host plants [3]. Although endophytes are associated with living tissues, they are not pathogenic in action and help host plants from different biotic and abiotic stresses by scavenging and regulating the damaging reactive oxygen species (ROS) [4,5] and therefore there is a need to harness their potentials as an alternative source of plant metabolites for development and synthesis of novel drugs.

Endophytic fungus remains a promising novel source of important bioactive compounds with therapeutic potential for the treatment of numerous diseases in pharmaceutical industries because of their ability to synthesize therapeutic biologically active agents with numerous pharmacological activities which are largely untapped. In our previous study, we reported the isolation, molecular identification and detection of bioactive constituents an Endophytic fungus *Fusarium equiseti,* which we isolated from the fresh leaf of *O. gratissimum*. [6] In this study, we report the isolation and identification of key bioactive constituents present in the extracts of the fungal endophyte. We also reported the detection of further bioactive constituents in fractions of the extracts to further highlight the biodiversity potential of the fungal endophyte, *Fusarium equiseti*.

2. Material and methods

2.1. Isolation, identification and fermentation of endophytic fungus

The isolation, molecular identification and fermentation of the fungal endophyte, *Fusarium equiseti,* was previously described [6].

2.2. Vacuum Liquid Chromatography

This was carried out as discussed in our previous report [6]. The collected fractions were used for other chromatographic and biological analyses.

2.3. Gel Permeation Chromatography

Fractions obtained from VLC were subjected to gel chromatography on sephadex LH-20. The fractions include n-hexane/ethyl acetate 50:50, Dichloromethane/methanol 80:20, and Dichloromethane/methanol 95:5b. The dry gel medium was dissolved in an excess solution of methanol and swollen for at least 3 hours. The media slurry was then poured down in a continuous motion down the side of a glass rod to avoid the introduction of air bubbles into the media. The gel media was allowed to gently pack as the outlet was opened, allowing equilibration of the incoming mobile phase. After a little while, a uniformly packed bed of constant height was achieved. The sample dissolved in little quantity of the mobile phase DCM/MeOH 50:50 was applied above the packed gel and allowed to completely absorb into the gel. Enough volume of the mobile phase consisting of DCM/MeOH 50:50 was gently poured through the sidewall of the column to avoid disrupting the column bed. As solvent passes continuously through the column, molecules that are larger than the pores of the matrix are unable to diffuse into the pores and elute through the column while the smaller molecules diffuse into the pores and their passage down the column is further delayed. The molecules move in that steady manner according to their molecular weights until all have been eluted from the column. Properly labeled test tubes were placed serially and little fractions of the eluate up to 2 ml were collected for each of the samples to give 53 fractions. The fractions were pooled by TLC analysis to obtain fractions A1 to A12.

2.4. Semi-preparative HPLC

Semipreparative purification of fraction A7 (n-Hexane/EtAc 50:50 subfraction) obtained from gel chromatographic separation was accomplished on a Merck Hitachi system consisting of an L-7400 UV detector and an L-7100 pump connected with a Kipp & Zonen flatbed recorder. The sample was prepared at a concentration of 3 mg/ml and 100 µl of the sample was applied into the sample loop at each injection. The mobile phase used is a mixture of polar solvents; including methanol, and water (nanopure water), and the flow rate was 5 mL/minute. The composition of the mobile phase was continuously changed from more to less polar conditions (gradient elution). Trifluoroacetic acid (0.1 %) was

added to water to get a high resolution of separated peaks. The attached column was a Knauer VertexPlus C18 column (Eurospher 100-10, 300 × 8 mm, L × i.d.). An online chromato-integrator was connected to the detector to monitor the individual components when they leave the column after separation. The peaks of the chromatogram account for the number of components eluted by the solvent system and collected in various sample collectors. This led to isolation of compounds A7-S1 to A7-S6.

2.5. Analytical HPLC-DAD

Analytical HPLC was used to identify important peaks in the fractions as well as to evaluate the purity of isolated compounds. Fractions A7 and A8 as well as the isolated compounds A7-S1 to A7-S6 were subjected to HPLC-DAD analysis. The HPLC analysis was performed using a Dionex Ultimate 3000 System coupled to a photodiode array detector (DAD300RS). The separation column used was (125 × 4 mm, L × i.d.) prefilled with Eurospher-10 C18. Each of the dried fungal fractions (2 mg) was reconstituted with 2 mL of HPLC grade methanol. The mixture was sonicated for 10 min and thereafter centrifuged at 3000 rpm for 5 min. Then, 100 µl of the dissolved samples were each transferred into HPLC vials containing 500 µl, of HPLC grade methanol. The gradient elution used started from 10:90% MeOH: nanopure H_2O (0.1% formic acid) to 100% MeOH in 60 minutes. Peaks were detected at 235, 254, 280, and 340 nm, and known substances were identified by comparison of the obtained UV spectra with the internal UV-spectra library using the online software.

3. Results

3.1. Thin Layer Chromatography of the sephadex fractions

The gel permeation chromatography led to a collection of fifty-three fractions with the presence of likely diversely colored secondary metabolites. Thin-layer chromatography of collected fractions afforded twelve fractions after bulking up in accordance with their band patterns. The Rf values and number of spots obtained in each fraction of the bulks are presented in Table 1. TLC data provides an idea of the compounds/substances present in a crude extract. The separated bands' colours are viewed under ultraviolet (UV) light at 254 nm and their rf values indicate the secondary metabolites likely present in the fractions when eluted with authentic standards.

Table 1 TLC experimental of the Sephadex subfractions of fractions S3, S5 & S7

Fraction	Substance separated	Number of spots	Dist traveled by spot (mm)	Dist traveled by solvent front (mm)	Rf
A1	DM 1-4	0	0	46	
A2	DM 5-16	4	5	44	0.11
A3	DM 17-24	2	21, 30	45	0.47, 0.66
A4	DM 25-47	3	4.6, 10.3, 13.7	40	0.11, 0.26, 0.34
A5	EH 3-14	0	0	51	
A6	EH 15-22	3	22.5, 33, 37.5	45	0.5, 0.73, 0.83
A7	EH 23-30	4	30, 36, 49, 51	51	0.58, 0.7, 0.96, 1
A8	EH 31-42	1	40	63	0.63
A9	#DM 4-14	0	0	52	
A10	#DM 15-19	3	25, 29, 34	52	0.48, 0.56, 0.65
A11	#DM 20-25	4	18.6, 21.4, 25.7, 30	50	0.37, 0.43, 0.51, 0.6
A12	#DM 26-37	2	16, 20	42	0.38, 0.48

Key: DM: Dichloromethane/Methanol (80:20) spotted fraction, EH: Ethyl acetate/n-hexane (50:50) spotted fraction, #DM: Dichloromethane/Methanol (95:5) spotted fraction.

3.2. Analytical HPLC-DAD of fractions A7 and A8

The chromatogram, UV spectrum, and molecular structures of the compounds detected are presented in Figures 1 and 2. The chromatogram represented in figure 6 showed the presence of six compounds namely: enniatin A, and aureonitol,

serasinoside H1, altenusin, aplysinamisin-1, and an isomer of cyclopenol and cyclopenin. Furthermore, the chromatogram in figure 7 revealed the presence of three (3) compounds A, B, and C with 3 different peaks which includes a benzylnitril (an organic precursor), ruspolinone (a pyrrolidine alkaloid), orientin (a flavone glycoside) which have been reported for antioxidant activity.

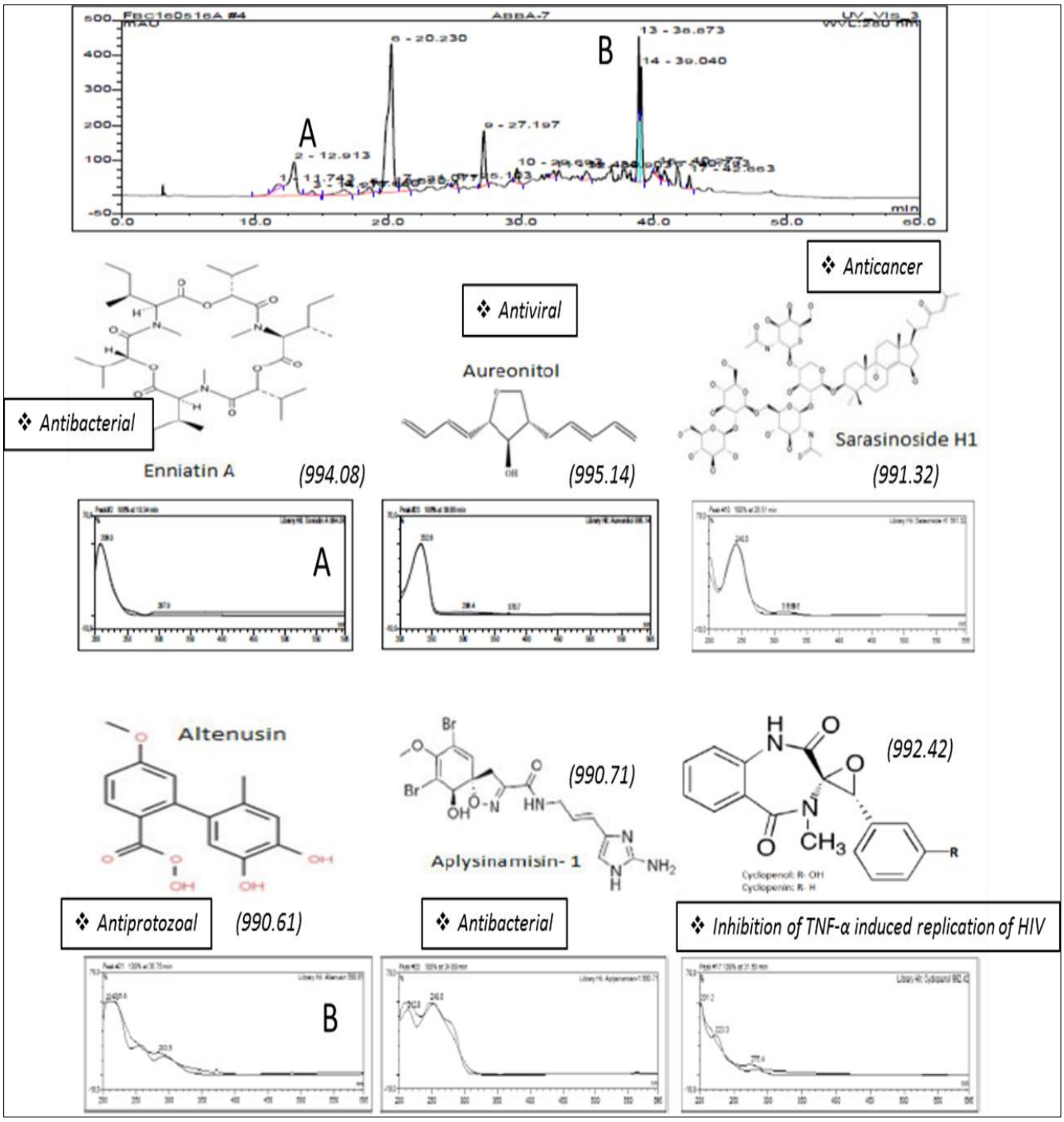


Figure 1 HPLC Chromatogram and UV absorption spectra analytical HPLC showing compounds such as enniatin A, aureonitol, serasinoside H1, altenusin, aplysinamisin-1, and an isomer of cyclopenol and cyclopenin isolated

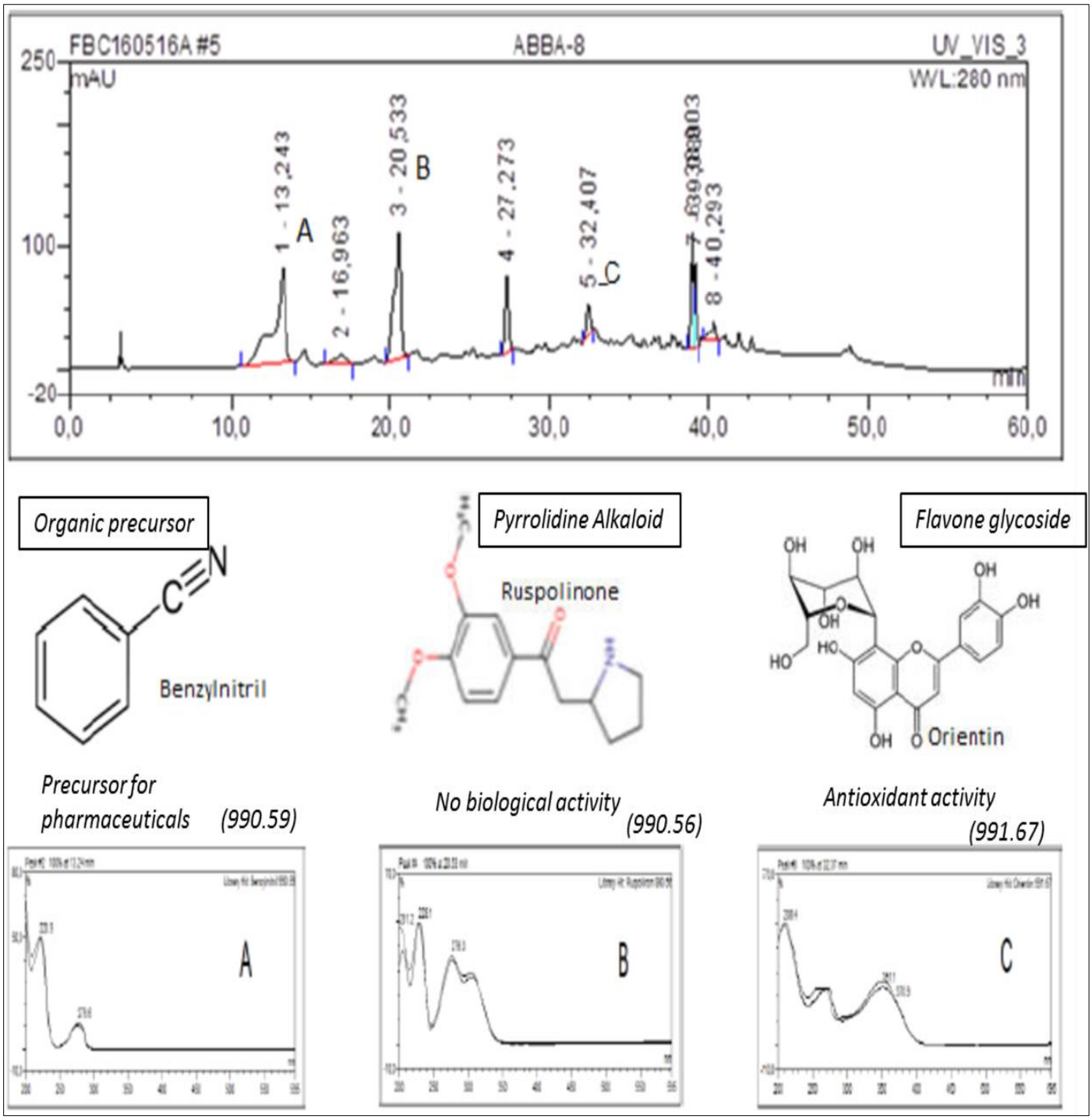


Figure 2 HPLC Chromatogram and UV absorption spectra analytical HPLC of showing the presence of compounds such as benzylnitril, and ruspolinone orientin. Identified

3.3. Semi-preparative HPLC

Semi-preparative HPLC of fraction A7 yielded six different compounds. The compounds with their observable characteristics are shown in Table 2 & Figures 3-7.

Only the compounds A7-S1 and A7-S2 were identified as ruspolinone and benzylnitril respectively by dereplication. The other compounds did not show strong library hits.

Table 2 Table showing compounds isolated from fraction A7 and associated characteristics

	Isolated compounds	Quantity (mg)	Characteristics
1	A7-S1	0.25	White crystalline
2	A7-S2	0.1	Colourless liquid
3	A7-S3	0.25	White solid
4	A7-S4	0.146	White crystalline
5	A7-S5	0.30	Colourless oil
6	A7-S6	0.5	Colourless oil

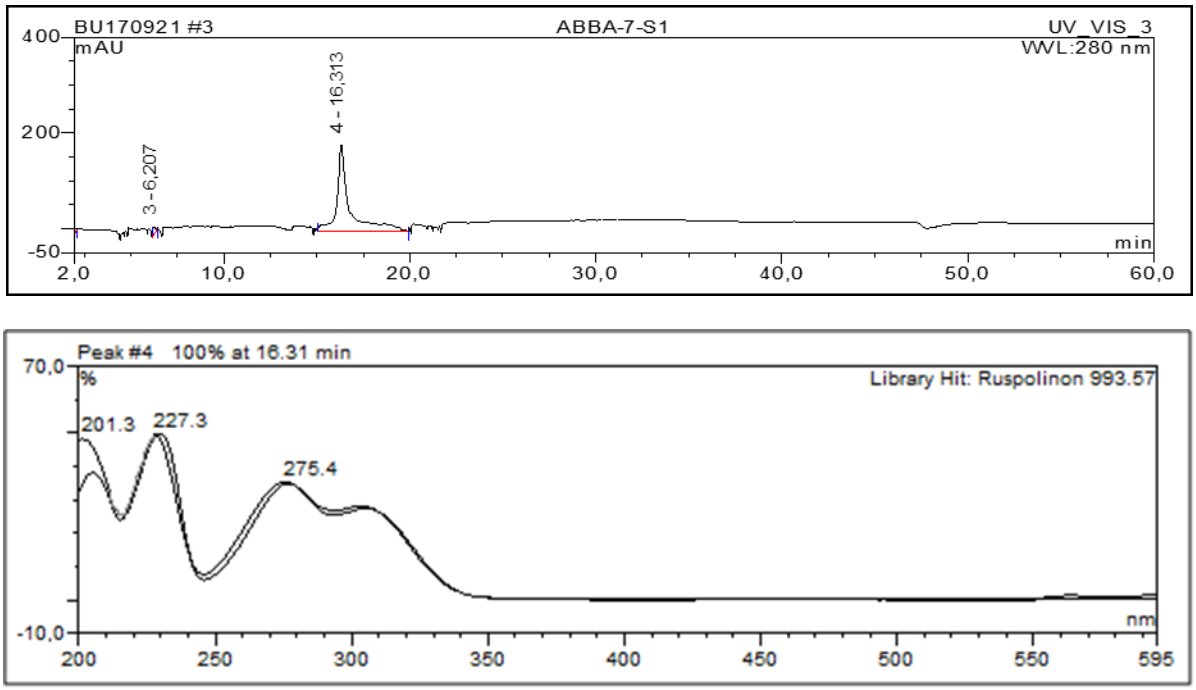
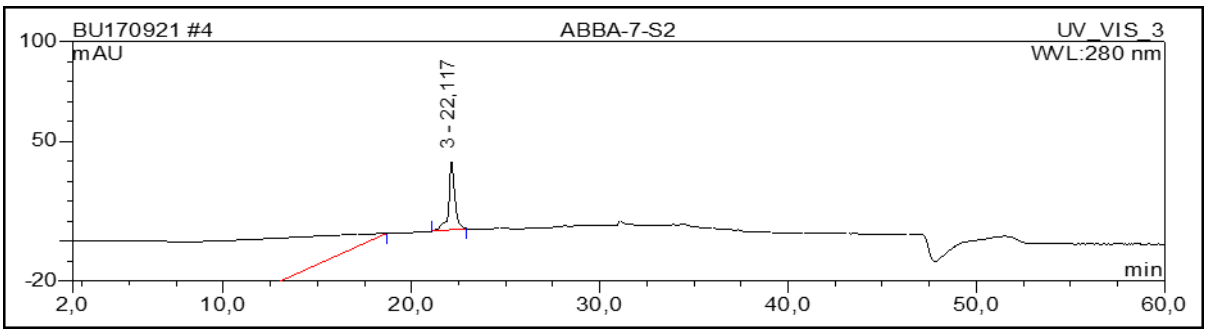


Figure 3 HPLC Chromatogram and UV absorption spectra of Semipreparative HPLC showing a compound identified as Ruspolinon



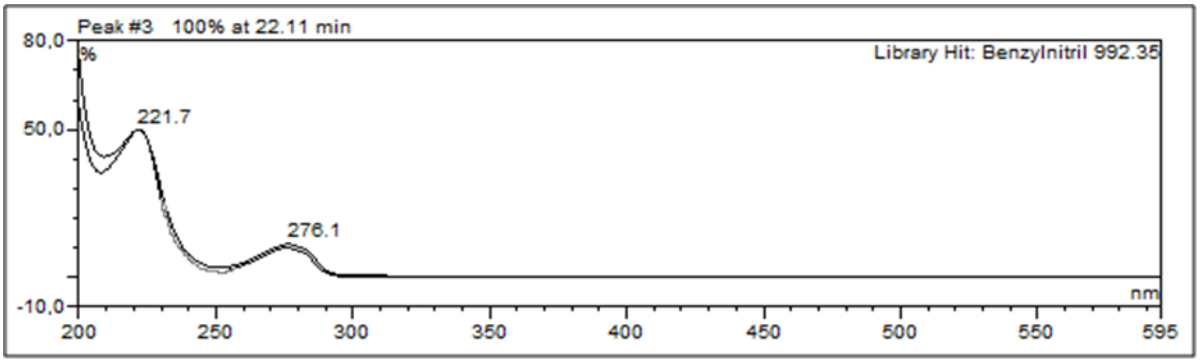


Figure 4 HPLC Chromatogram and UV absorption spectra of Semipreparative HPLC showing a compound identified as Benzynitril

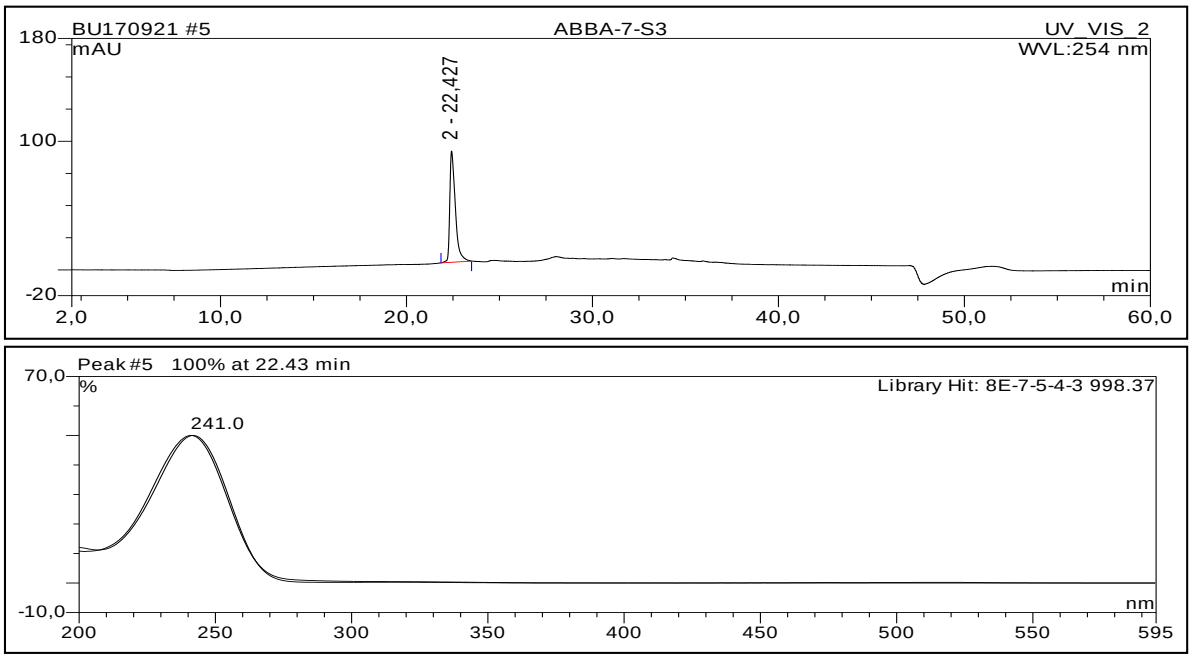


Figure 5 HPLC Chromatogram and UV absorption spectra of Semipreparative HPLC showing an unidentified compound (Potential active compound under development)

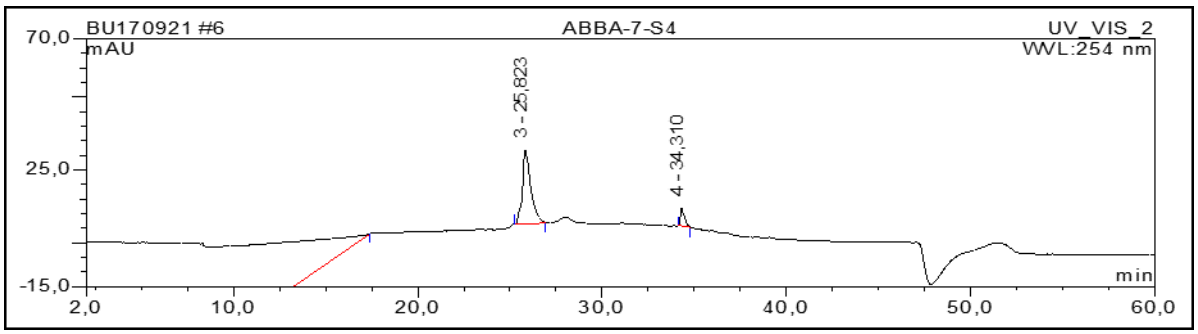


Figure 6 Chromatogram spectra of Semipreparative HPLC showing an unidentified compound (Potential active compound under development)

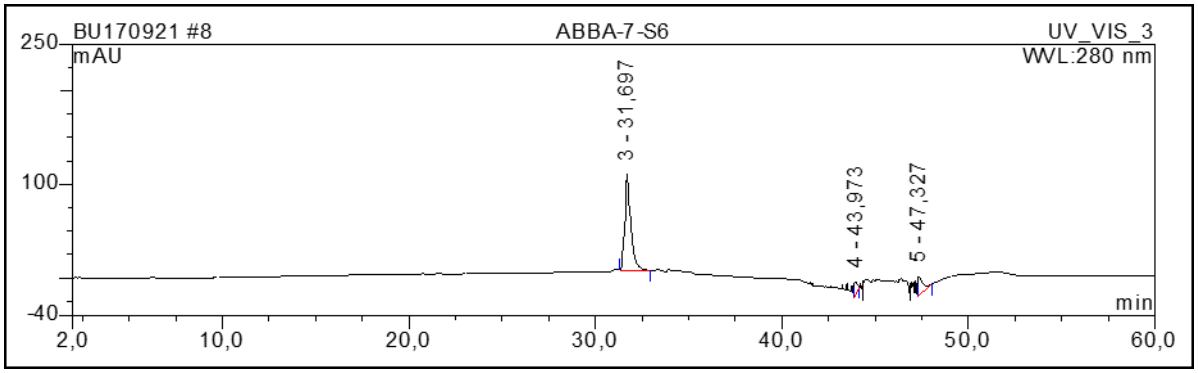


Figure 7 Chromatogram spectra of Semipreparative HPLC showing an unidentified compound (Potential active compound under development)

4. Discussion

Numerous bioactive compounds with apparent biological activity, including antiviral, antifungal, hepatoprotection, antibacterial, anticancer, cytotoxic, and antioxidant capabilities, were detected from the Endophytic fungi, *Fusarium equiset* in our previous report [6]. Because of the large spectrum of chemicals with various biological activities contained in the extract of this Endophytic fungus we further investigated the fractions and identified Enniatin A, Aureonitol, Serasinoside H1, Altenusin, Aplysinamisin, benzylnitril, ruspolinone and Orientin. These compounds have been shown to exhibit different biological activities in previous studies.

Enniatins are a group of organic compounds identified in many *Fusarium sp* and have been shown to exhibit apoptotic characteristics and antibacterial activity against some pathogens of humans, such as *E. coli, E. faecium, S. enterica, S. dysenteriae, L. monocytogenes, Y. enterocolitica, C. perfringens, P. aeruginosa*, and strains of *S. aureus* [7]. Enniatins have also been reported in previous studies of [8-9].

Aureonitol is a tetrahydrofuran derivative previously isolated from mycelium plugs of *Chaetomium globosum* and *Chaetomium coarctatum* with antiviral properties [10]. Aureonitol was reported to inhibit the Influenza virus (A and B) by binding to surface hemagglutinin and impairing virus adsorption [10].

Sarasinoside H1 is a saponin-containing amino sugar isolated from spongy marine living forms that has been shown to have potent cytotoxicity against a variety of tumor cells as well as inhibit protein kinase C [11].

Cyclopenol is a naturally occurring 7-membered 2,5-dioxopiperazine alkaloid that was first isolated from an Endophytic fungus, *Penicillium cyclopium*, from the Chinese mangrove *Bruguiera gymnorrhiza* [12]. It was also isolated from the Endophytic fungus *Daldinia eschscholtzii*, isolated from the leaves of *Musa paradisiaca* [13]. Altenusin previously isolated from the Endophytic fungus *Alternaria sp*. was reported to have strong antifungal activity against clinical isolates of *P. brasiliensis* [14] and also inhibited trypanothione reductase from *Trypanosoma cruzi*, a neglected tropical disease [15]. Aplysinamisin is an isoxazoline alkaloid found in the *Aplysinia aerophoba* and *Aplysina cavernicola* marine sponges [16].

The chromatogram in figure 2 revealed the presence of benzynitril, ruspolinone and orientin among an array of unidentified peaks. Benzylnitril is a colorless oily aromatic liquid that serves as a precursor to a variety of organic compounds. Neither benzylnitril nor ruspolinone have been linked to any biological action. However, Orientin a flavone glycoside has been isolated in numerous plants such as *Ocimum sanctum* [17-19], *Passiflora species* [20, 21], and *Jatropha gossypifolia* [22-25]. Orientin has also been reported to exhibit antioxidant [26, 27], antiviral [28], and antibacterial activity against *Escherichia coli, Staphylococcus aureus, Staphylococcus cohnii, Klebsiella pneumoniae*, and Proteus bacteria [29]. It has also been reported to exhibit anti-inflammatory [30, 31] properties.

We attempted further purification of one of the very promising fractions using a combination of chromatographic techniques including gel chromatography on Sephadex and semi-preparative HPLC. Unfortunately, the compounds were isolated in very minute quantities, which was not enough for detailed NMR spectral analysis. Two of the compounds were, however, identified by dereplication using HPLC-DAD as Ruspolinone and benzinitril. The others

were not identified due to lack of library hits. Further structural analysis of these compounds is envisaged in our laboratory since they have great potential of turning out new and novel bioactive lead compounds.

5. Conclusion

Further investigation of fractions from the Endophytic fungus *Fusarium equiseti* let to detection and identification of many more bioactive compounds with promising biological activity, such as antiviral, antifungal, antibacterial, anticancer, cytotoxic, and antioxidant characteristics. This Endophytic fungus, *Fusarium equiseti,* isolated from *O. gratissimum* should thus be exploited for future pharmacological, pharmaceutical, agricultural and industrial uses.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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Authors Contribution statement

All the authors contributed in the conceptualization of the research, Peter Eze and Nnamdi contributed in the data collection, Onyeka Ifeanyi Peter, Nkeoma Nkasi Okoye, Ogechukwu Anyanwu and Blessing Umeokoli contributed in the analysis and development of the first draft of the manuscript while Professor F.B.C Okoye supervised the work with Dr Abba Chika as co-supervisor. Professor FBC Okoye reviewed the final manuscript.

Data Availability Statement

Data generated or analyzed during this study are provided in full within the published article and its supplementary materials.

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